BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

B25

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 August 2000 (31.08.2000)

PCT

(10) International Publication Number
WO 00/50644 A3

(51) International Patent Classification[7]: C12Q 1/68, G01N 1/34, B01L 3/02 // B01J 19/00

(21) International Application Number: PCT/US00/04939

(22) International Filing Date: 25 February 2000 (25.02.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/259,467 26 February 1999 (26.02.1999) US

(71) Applicant: MOSAIC TECHNOLOGIES [US/US]; 303 Bear Hill Road, Waltham, MA 02451 (US).

(72) Inventors: ADAMS, Christopher, P.; 141 Powderhouse Boulevard, Somerville, MA 02144 (US). BOLES, T., Christian; Five Judith Lane #6, Waltham, MA 02451 (US). WEIR, Lawrence; 55 Granite Street, Hopkinton, MA 01748 (US). DHANDA, Rahul, K.; 27 Linden Avenue #2, Somerville, MA 02143 (US). SUMMERS, Nevin, M.; 75 Monmouth Street, Brookline, MA 02446 (US).

(74) Agents: HOGLE, Doreen, M. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).

(81) Designated States *(national)*: AU, CA, JP.

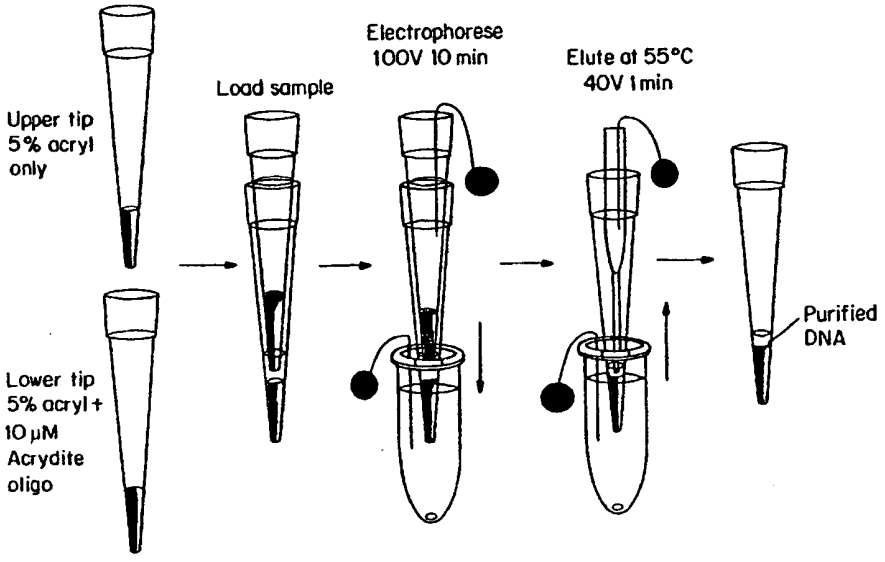
(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*

(88) Date of publication of the international search report:
5 April 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS FOR PURIFYING DNA USING IMMOBILIZED CAPTURE PROBES



(57) Abstract: Methods employing purification devices comprising an electrophoretic medium containing immobilized capture probes for the purification of DNA are disclosed.

WO 00/50644 A3

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/US 00/04939

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 G01N1/34 B01L3/02 //B01J19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q B01J G01N B01L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94 20831 A (WAINWRIGHT NORMAN ;BOYD STEVEN H (US)) 15 September 1994 (1994-09-15) page 1, paragraph 4 -page 2, paragraph 2 page 2, paragraphs 4,5 page 2, paragraph 7 -page 3, paragraph 3 page 5, paragraph 2 page 6, line 23 -page 6, line 31 examples 4,6-8 figures 1-3 --- -/--	1-6,8, 10-17, 19,20, 22-24, 26,27

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 2000	29/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Koch, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/US 00/04939

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 639 603 A (NEEDELS MICHAEL C ET AL) 17 June 1997 (1997-06-17) column 17, line 42 -column 17, line 53	1-6,8, 10-17
Y	column 18, line 4 -column 18, line 12 column 18, line 28 -column 18, line 53 column 19, line 47 -column 20, line 46 column 21, line 17 -column 22, line 7 column 30, line 50 -column 30, line 65 column 36, line 10 -column 37, line 6 column 39, line 4 -column 39, line 14	19,20
Y	column 42, line 27 -column 42, line 59 column 43, line 41 -column 43, line 56 column 44, line 14 -column 44, line 26 figure 1	22-24, 26,27
A	US 5 560 811 A (MCCORMICK RANDY M ET AL) 1 October 1996 (1996-10-01) column 7, line 62 -column 8, line 16 column 8, line 55 -column 9, line 4 column 9, line 24 -column 10, line 23 column 10, line 46 -column 10, line 63 column 11, line 1 -column 11, line 16 column 11, line 43 -column 11, line 52 column 13, line 33 -column 14, line 19 column 14, line 38 -column 14, line 67 figures 1-9 column 9	1-18
A	US 5 556 598 A (KENRICK MICHAEL K ET AL) 17 September 1996 (1996-09-17) column 1, line 5 -column 1, line 14 column 1, line 38 -column 2, line 4 column 3, line 9 -column 3, line 20 column 3, line 30 -column 3, line 43 column 4, line 54 -column 4, line 64 column 5, line 54 -column 6, line 52	1,5-8, 19,20
Y	column 7, line 17 -column 7, line 67 figures 1-6	6,15

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internati Application No
PCT/US 00/04939

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9420831 A	15-09-1994	AU 6360994 A	26-09-1994
		EP 0688426 A	27-12-1995
		US 5876918 A	02-03-1999
US 5639603 A	17-06-1997	US 5770358 A	23-06-1998
		AU 703472 B	25-03-1999
		AU 1128095 A	23-05-1995
		BR 9407947 A	26-11-1996
		CN 1134156 A	23-10-1996
		EP 0726906 A	21-08-1996
		GB 2298863 A,B	18-09-1996
		JP 9508353 T	26-08-1997
		NZ 276860 A	22-09-1997
		WO 9512608 A	11-05-1995
		US 6140493 A	31-10-2000
		AT 148889 T	15-02-1997
		AU 669489 B	13-06-1996
		AU 2661992 A	27-04-1993
		CA 2118806 A	01-04-1993
		DE 69217497 D	27-03-1997
		DE 69217497 T	12-06-1997
		DK 604552 T	04-08-1997
		EP 0604552 A	06-07-1994
		EP 0773227 A	14-05-1997
		ES 2097925 T	16-04-1997
		GR 3023156 T	30-07-1997
		US 6143497 A	07-11-2000
		WO 9306121 A	01-04-1993
		US 5789162 A	04-08-1998
		US 5708153 A	13-01-1998
US 5560811 A	01-10-1996	AU 715852 B	10-02-2000
		AU 5318796 A	08-10-1996
		CA 2215974 A	26-09-1996
		EP 0815440 A	07-01-1998
		JP 11502618 T	02-03-1999
		WO 9629595 A	26-09-1996
US 5556598 A	17-09-1996	CA 2105962 A	19-03-1994
		DE 69305947 D	19-12-1996
		DE 69305947 T	13-03-1997
		EP 0588564 A	23-03-1994
		JP 6315603 A	15-11-1994
		US 5833927 A	10-11-1998

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68

A2

(11) International Publication Number: **WO 00/50644**

(43) International Publication Date: 31 August 2000 (31.08.00)

(21) International Application Number: PCT/US00/04939

(22) International Filing Date: 25 February 2000 (25.02.00)

(30) Priority Data:
09/259,467 26 February 1999 (26.02.99) US

(71) Applicant: MOSAIC TECHNOLOGIES [US/US]; 1106 Commonwealth Avenue, Boston, MA 02215 (US).

(72) Inventors: ADAMS, Christopher, P.; 141 Powderhouse Boulevard, Somerville, MA 02144 (US). BOLES, T., Christian; Five Judith Lane #6, Waltham, MA 02451 (US). WEIR, Lawrence; 55 Granite Street, Hopkinton, MA 01748 (US). DHANDA, Rahul, K.; 27 Linden Avenue #2, Somerville, MA 02143 (US). SUMMERS, Nevin, M.; 75 Monmouth Street, Brookline, MA 02446 (US).

(74) Agents: HOGLE, Doreen, M. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHODS FOR PURIFYING DNA USING IMMOBILIZED CAPTURE PROBES

(57) Abstract

Methods employing purification devices comprising an electrophoretic medium containing immobilized capture probes for the purification of DNA are disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

METHODS FOR PURIFYING DNA USING IMMOBILIZED CAPTURE PROBES

BACKGROUND OF THE INVENTION

Nucleic acid sequence information plays a vital role in both basic and applied biomedical research. The nucleotide sequence of a particular portion of DNA can be instructive as to the molecular basis for a given disease, such as Huntington's Disease. Once a segment of genome has been identified as being potentially responsible for a particular affliction, elucidating the nucleotide sequence becomes very important. The sequence, once known, can play a part in the therapeutic regime to be provided, such as in the case of gene therapy. This is most evident when the basis of the disease is a genetic mutation of the normal gene. One methodology employed for treating genetic mutation-based diseases is the introduction of the wild-type nucleotide sequence. But first it must be established that in fact a gene, or an aberrant form of a gene, is the etiologic agent for a particular disease or syndrome. This information is most often provided through the isolation and characterization of the putative aberrant gene. Characterization often involves the sequence analysis of the nucleotide sequence itself that defines the gene of interest. This will often involve understanding both the wild-type, or physiologically normal, and mutant genes.

In practice, the quality of the sequence analysis is, in part, a reflection of the quality of the starting material. It is vital that the preparation that is to be subjected to sequence analysis be of high quality, that is, relatively pure and free of

contaminating species like proteins and small molecules, such as salts, that can interfere with obtaining a high quality result from sequence analysis. Current protocols involve ethanol precipitation in order to remove unincorporated nucleotides and salt from, for example, extension products prior to sequence analysis. Also, with regard to the extension products, it is often desirable to remove any template DNA and excess primers from the preparation prior to sequence analysis. Precipitation is time consuming and requires care to achieve consistent product yields. Nevertheless, it is critical to the success of performing an informative sequence analysis on a target nucleotide sequence that the target sequence preparation be as free of contaminating molecules as possible. Additionally, it would be advantageous to have a purification system that could sort the products of multiplexed sequencing reactions.

SUMMARY OF THE INVENTION

The present invention pertains to methods of purifing a target molecule contained within a test sample. Typically, the target molecule in a test sample will be a nucleic acid molecule, in particular, single-stranded DNA primer extension sequencing reaction products of a dideoxy sequencing reaction, for example, the Sanger method, Sanger, F., *et al.*, *Proc. Natl. Acad. Sci. USA*, 74:5463-5467 (1977), or a cycle sequencing method, Carothers, *Biotechniques*,, 7:494-499 (1989), the entire teachings of which are herein incorporated by reference in their entirety. Once purified, these purified nucleic acid molecules can be used in a variety of ways including being subjected to capillary or slab gel electrophoresis for DNA sequence analysis. Nucleic acid molecule capture probes modified with 5'-acrylamide groups are copolymerized within an electrophoresis gel, such as a polyacrylamide gel. Single-stranded target nucleic acid molecules can bind to their complementary sequence contained within a capture probe, if there is sufficient complementarity between the two molecules. Double-stranded target nucleic acid molecules can bind to a complementary sequence forming a triple helical arrangement.

The target molecule, or molecules, present in a test sample can be placed in an electrophoresis gel containing immobilized capture probes and undergo electrophoresis. The target molecule will migrate through the gel medium until it

comes in contact with its complementary immobilized capture probe. Once the target and capture probe are in contact with one another, they can hybridize forming a complex. The non-target molecules contained in the test sample can continue electrophoresis and are effectively removed from the target molecule.

In one embodiment of the present invention, the target molecules are DNA extension products formed during a primer extension sequencing reaction. A reaction mixture from a primer extension sequencing reaction is loaded into a purification device, for example, a microtiter plate containing multiple wells (having, e.g., 6, 12, 96 or 384 wells). The purification device comprises an electrophoresis gel containing immobilized capture probes that are complementary to at least one nucleotide sequence region contained within the target molecules. Preferably, an electric field is applied such that all negatively charged molecules migrate through the electrophoresis gel toward the a positively charged electrode. The positively charged electrode can be housed in a positively charged electrode buffer chamber. This chamber can be used to collect molecules that exit the electrophoretic medium as a result of their electrophoretic migration. The target molecules will be captured by complementary, immobilized capture probes that are within the gel. The non-target molecules contained within the test sample will pass through the gel and into the positively charged electrode buffer (also referred to herein as the collecting chamber). The collecting chamber can then be replaced with fresh positively charged electrode buffer. A sufficient voltage can be applied so as to denature the hybridization complex formed between the target molecule and capture probe thereby releasing the target molecule. The electric field can be applied using the same polarity as originally applied, thereby allowing for the continued migration of the released target molecule into the collecting chamber containing fresh positively charged electrode buffer. Alternatively, the electric field can be reversed drawing the released target molecule back into the test sample well of the purification device. The purified target molecule can now be accessed and subjected to further analysis, such as capillary or slab gel electrophoresis for sequence analysis.

Another embodiment of the invention is a kit for purifying a primer extension sequencing reaction. The kit contains an electrophoretic medium which

has a capture probe, or a set of capture probes. At least one capture probe has a sequence of at least 5 nucleotides in length which is substantially complementary to a portion of at least one primer extension sequencing reaction product.

In a further embodiment, the kit contains multiple electrophoretic media for purifying multiple primer extension sequencing reactions. Each electrophoretic medium in the kit contains a capture probe, or set of capture probes. At least one capture probe in each medium has a sequence of at least 5 nucleotides in length which is substantially complementary to a portion of at least one primer extension sequencing reaction product.

Using the method of the invention primer extension sequencing reaction products can be purified directly without time consuming concentration or precipitation steps typically required in current protocols for DNA sequencing. In addition, the method of the invention can be used to sort out primer extension sequencing reaction products from multiple primer extension sequencing reactions carried out in the same reaction mixture, by selecting the appropriate capture probes. Thus, the method of the invention allows many primer extension sequencing reactions to be carried out together saving preparation time required to set up multiple separate sequencing reactions.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of purifying a target nucleic acid molecule from a extension sequencing reaction using an electrophoresis gel with capture probes immobilized within a region of the gel.

Fig. 2 is a schematic representation of the steps involved in purifying extension products using a microtiter well comprising an electrophoretic medium containing capture probes immobilized within the medium. In Fig. 2, "ddNTPs" represents dideoxynucleotide triphosphates, "pol" represents a DNA polymerase, and "ex. products" represents primer extension sequencing reaction products.

Fig. 3 is the organization of the forward primer (SEQ ID NO 1), the template (SEQ ID NO 2) and the capture probe (SEQ ID NO 3) used in Example 1.

Fig. 4 is a schematic drawing illustrating the experimental design for DNA isolation using an electrophoretic medium.

Fig. 5 is shows the effects of varying the elution voltage.

Fig. 6 is shows results obtained from subjecting extension sequencing reaction products to electrophoresis in which the electrophoretic medium contained immobilized capture probes; Fig. 6A shows the results of the experiment after running the gel for thirty minutes; Fig. 6B shows the results of the experiment after sixty minutes.

Fig. 7A is a schematic representation of a device and a method used to purify the primer extension sequencing reaction products of Example 2.

Fig. 7B shows the distribution of the primer extension sequencing reaction products in the lower gel-tip which contained the capture probe.

Fig. 8 is a photograph of an electrophoresis gel showing the distribution of component of a primer extension sequencing reaction taken prior to purification (Lane 1), after purification using with the upper gel-tip alone (Lane 2), and after purification with the lower gel-tip containing a capture probe (Lane 3). Lanes 4-6 contain purified M13 DNA of varying concentrations.

Fig. 9A is a schematic representation of forward and reverse primer extension sequencing reactions carried out simultaneously in Example 3.

Fig. 9B is an image of the forward and reverse primer extension sequencing reaction product separated using two gel-tips. The upper gel-tip contains a capture probe complementary to the forward primer extension sequencing reaction products. The lower gel-tip contanins a capture probe complementary to the reverse primer extension sequencing reaction products.

Fig. 9C is an image of the separation of the products of a forward and reverse primer extension sequencing reaction product on a slab gel which contained two capture probes (Lane 1). The purity of the reverse primer extension sequencing reaction products after separation from the forward primer extension sequencing reaction products is shown in Lane 2. One capture probe was complementary to the reaction products of the forward sequencing reaction and the other capture probe was complementary to the reaction products from the reverse sequencing reaction.

Fig. 9D is an analysis of the reverse sequencing reaction products after purification by the method of the invention.

Fig. 10 is a temperature gradient gel used to determine the temperature at which a target is released from a capture probe.

Fig. 11 is an image of a gel used to determine the temperature at which a target will be released from capture probes of varying lengths.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides both methods and devices employed to purify a test sample containing a target molecule. It should be understood that the use of the singular term "target molecule" is only used for simplicity throughout this application, and the plural form "target molecules" is implied therein. The methods described herein employ nucleic acid molecule (e.g., oligonucleotide) capture probes immobilized within an electrophoretic medium. The capture probe can be dispersed throughout the electrophoretic medium, or immobilized within discrete layers of the medium as described in U. S. Serial No. 08/971,845, the entire teaching of which is incorporated herein by reference in its entirety. This electrophoretic medium is contained within a purification device, such as a microtiter plate. The capture probes can be designed to specifically interact with, and hybridize to, a target molecule contained within a test sample. The test sample comprising target and non-target molecules can be introduced into the device, for example, a microtiter plate comprising an electrophoretic medium containing immobilized capture probes. An electric field can be applied to the purification device so that charged molecules in the test sample will migrate within the electrophoretic medium toward the appropriate pole. For example, for the device used in Example 1, voltages for capture would fall in the range of 0.1 to 200 V, more preferably, between 50 and 150 V. Typically, the molecules of interest will possess a negative charge and therefore migrate toward the positively charged electrode. The target molecule will continue to migrate until it has come into contact with an immobilized capture probe which is specific for that particular target molecule. A hybridization complex can then form between the immobilized capture probe and target molecule. (See Fig. 1). This hybridization complex prevents further migration of the target molecule and allows for the continued migration of non-target molecules, thereby effectuating purification of the target molecule contained within the test sample. Non-target

molecules can include proteins, such as enzymes, small molecules like salts, non-targeted nucleotides as well as other non-target molecules, that is, those molecules not targeted for further processing. (See Fig. 2). The target molecule can subsequently be released from the capture probe by applying a sufficient voltage or temperature and exit the gel for further analysis. For example, using the device used in Example 1, the target molecule can be eluted from the capture layer (i.e., that layer in the electrophoresis medium containing the immobilized capture probes) at voltages of 250V or higher. Preferably, voltages for elution would be in the range of 250 to 1000 V, more preferably, 250 to 300 V. Suitable voltages for capture and elution using the purification devices described herein can be easily determined by one of skill in the art. Examples 4 and 5 provide methods for determining the temperature at which the target molecules are released from capture probes of varying lengths.

The methods of the present invention use a purification device which comprises three regions. The first region comprises a test sample receptacle which receives a given test sample. The test sample receptacle can be positioned in such a manner as to be proximal to at least one orifice that allows for the delivery of a test sample (e.g., a reaction mixture from a primer extension sequencing reaction). In one embodiment, this orifice is the opening at the top of a microtiter well. The second region of the purification device comprises an electrophoretic medium. Preferably, the electrophoretic medium comprises capture probes immobilized within the medium. Preferably, this second region is physically positioned adjacent to the first region. In one embodiment, the second region is positioned basally to the first position and is also adjacent to the first region. In a preferred embodiment, this second region is formed within one, or more, microtiter wells, though still allowing for the first region to receive and store test sample. The third region of the purification device can be physically contiguous with, or attached to the second region of the purification device. This third region can house a chamber that can collect molecules that exit the second region, in this instance the chamber is referred to as a collecting chamber. The chamber can also perform other functions such as to house buffer. The purification device can also be attached to, or have the capacity to connect with, a power source which generates DC voltage (e.g., a battery).

In one embodiment, the purification device is a microtiter plate containing a set of multiple wells, for example 6, 12, 48, 96 or 384 wells. The well, or wells, of the microtiter plate comprises the three regions elucidated above for the purification device. The first region comprises a test sample receptacle for receiving test sample. The second region comprises an electrophoretic medium that contains immobilized capture probes. The third region can be formed by excising the bottom support of the well creating an orifice, in some instances the microtiter well has a pointed tip which can be trimmed to provide an opening. This bottom orifice can optionally be covered using a porous membrane to provide support for the gel layer (second region). Preferably, this porous membrane has a molecular weight cutoff greater than 15,000 daltons. More preferably, the molecular weight cutoff is approximately 3,000 daltons. Preferably, the porous membrane should demonstrate negligible binding of nucleic acid molecules. This bottom orifice can be used to gain access to the third region which can be physically attached or detached from the well itself. The third region can comprise a collecting chamber which can contain buffer.

Specifically encompassed by the present invention is a method for purifying primer extension sequencing reaction products from a primer extension sequencing reaction using a purification device described herein. The target molecule is one, or more, of the primer extension sequencing reaction products formed during a particular stage of a DNA sequencing protocol. Typically, the primer extension sequencing reaction product can have a size from about 20 to about 2000 nucleotides in length. For example, a DNA molecule that is destined for nucleotide sequencing can be placed into an appropriate sequencing vector, such as the M13 phage vector. Under suitable conditions well known to those skilled in the art, extension nucleic acid products can be produced using this vector, preferably using the cycle sequencing method. (See Fig. 1; Carothers, *Biotechniques*, 7:494-499 (1989), and Murray, *Nucleic Acid Res.*, 17:8889 (1989)). Some of the reactants employed in this primer extension sequencing reaction are DNA Polymerase, primers, deoxynucleotides, and appropriate salts. Those skilled in the art will be familiar with standard DNA sequencing protocols. (See, Ausbel, F.M., *et al.* (eds), Current Protocols in Molecular Biology, vol.1, ch.7, (1995)). The target molecule (primer

extension sequencing reaction product) can subsequently undergo purification using a purification device.

Another embodiment of the invention is a kit for purifying a primer extension sequencing reaction. The kit contains a electrophoretic medium which has a capture probe, or a set of capture probes. At least one capture probe has a sequence of at least 5 nucleotides in length which is substantially complementary to a portion of at least one primer extension sequencing reaction product. The kit can, optionally, include a sample receptacle and a collecting chamber. In one embodiment, the sample receptacle and the collecting chamber are located at opposite ends of the electrophoretic medium.

In a further embodiment, the kit contains multiple electrophoretic media for purifying multiple primer extension sequencing reactions. The electrophoretic media can be segregated from each other. Each electrophoretic medium in the kit contains a capture probe, or set of capture probes. At least one capture probe in each medium has a sequence of at least 5 nucleotides in length which is substantially complementary to a portion of at least one primer extension sequencing reaction product. Each electrophoretic medium can contain the same capture probe, or set of capture probes. This type of kit could be used to purify the reaction products from the same primer extension sequencing reaction which has been carried out on multiple samples in separate reaction vessels. Alternatively, the each electrophoretic medium in the kit can have a different capture probe or set of capture probes. This type of kit can be used to purify multiple primer extension sequencing reactions which are carried out in the same reaction vessel.

When the kit contains multiple electrophoretic media, each media can be segregated from other electrophoretic media in wells of a microtiter plate. Each electrophoretic medium can be attached to a separate sample receptacle and a separate collection chamber.

In one embodiment of the present invention, a method for purifying multiple primer extension sequencing reaction products which are formed by synthesizing target molecules (i.e., primer extension sequencing reaction products) using both a first-end (e.g., near or at the 5' end) and a second-end (e.g., near or at the 3' end) of the DNA template simultaneously is described. Primer extension sequencing can

occur in both directions of the template simultaneously. In this embodiment, a first-end primer and a second-end primer would be concurrently annealed to the template DNA allowing for extension in both direction using the one template DNA. The primer extension sequencing reaction would then occur and produce primer extension sequencing reaction products arising from both the first-end and the second-end of the DNA template. The primer extension sequencing reaction products can then be added to a purification device that can purify the target molecule based upon whether it was synthesized using the first-end primer or second-end primer.

In this embodiment, the purification device comprises at least two electrophoretic gel cartridges that can be brought together to form a continuity between the two cartridges. A gel cartridge is a device that can house and support an electrophoretic medium. The gel cartridges comprise electrophoretic medium containing capture probes immobilized within the medium. However, each cartridge comprises an electrophoretic medium containing different immobilized capture probes. For example, one cartridge can comprise an electrophoretic medium containing an immobilized capture probe that contains a nucleotide sequence which is substantially identical to a nucleotide sequence that lies adjacent, or close to, the first-end of the template (capture probe "A"), whereas, the second cartridge can comprise an electrophoretic medium which contains an immobilized capture probe that contains a nucleotide sequence which is substantially identical to a nucleotide sequence that lies adjacent, or close to, the second-end of the template (capture probe "B"). By "substantially identical to," it is meant a nucleotide sequence with greater than 70% sequence identity and/or similarity (e.g., 75%, 80%, 85%, 90%, or 95% or greater homology). Initial search for substantially identical nucleotide sequences can be performed at NCBI against the GenBank (release 87.0), EMBL (release 39.0), and SwissProt (release 30.0) databases using the BLAST network service. Altshul, S.F., *et al.,* Basic Local Alignment Search Tool, *J. Mol. Biol.*, 215:403 (1990), the entire teachings of which are incorporated herein by reference in its entirety. Computer analysis of nucleotide sequences can be performed using MOTIFS and the FindPatterns subroutines of the Genetics Computing Group (GCG, version 8.0) software. Nucleotide comparisons can also be performed according to

Higgins and Sharp (Higgins, D.G. and P.M. Sharp, Description of the Method used in CLUSTAL, *Gene*, 73:237-244 (1998)).

The two cartridges can be positioned in such a way as to allow for the migration of target molecules through one cartridge into the next cartridge.

For example, the cartridge that has the "A" capture probe is positioned such that it first receives the sample, and the second cartridge, which has the "B" capture probe, is positioned to receive the migrating sample from the first cartridge. If a test sample containing a heterogeneous mixture of target molecules (those that were synthesized using the first-end primer together with those that employed the second-end primer) is added to this purification device, then the target molecules can be purified or separated based upon the primer used to synthesize the target molecule. When an electric field is applied to the purification device, the target molecules in the test sample can undergo electrophoretic migration. Those target molecules that used the first-end primer for synthesis will be captured in the first cartridge containing "A" as capture probes (its appropriate capture probe), while those target molecules that used the second-end primer will migrate through the first cartridge and will subsequently be captured in the second cartridge containing "B" capture probes (its appropriate capture probe). Following electrophoretic migration, the cartridges can be separated and placed into separate collecting chambers, thereby allowing for the collecting of the first-end primer target molecules and the second-end primer target molecules separately.

Any electrophoretic matrix suitable for electrophoresis can be used for the methods of the present invention. Suitable matrices include acrylamide and agarose, both commonly used for nucleic acid electrophoresis. However, other materials may be used as well. Examples include chemically modified acrylamides, starch, dextrans, cellulose-based polymers. Additional examples include modified acrylamides and acrylate esters (for examples see Polysciences, Inc., Polymer & Monomer catalog, 1996-1997, Warrington, PA), starch (Smithies, *Biochem. J.*, 71:585 (1959); product number S5651, Sigma Chemical Co., St. Louis, MO), dextrans (for examples see Polysciences, Inc., Polymer & Monomer Catalog, 1996-1997, Warrington, PA), and cellulose-based polymers (for examples see Quesada, *Current Opin. in Biotechnology, 8:*82-93 (1997)). Any of these polymers listed

above can be chemically modified to allow specific attachment of capture probes for use in the present invention.

The capture probes of the instant invention are typically nucleic acids, modified nucleic acids, or nucleic acid analogs. The capture probes are complementary to the primer extension sequencing reaction products, but not to the primer extension sequencing primer. Methods of coupling nucleic acids to create nucleic acid-containing gels are known to those of skill in the art. Nucleic acids, modified nucleic acids and nucleic acid analogs can be coupled to agarose, dextrans, cellulose, and starch polymers using cyanogen bromide or cyanuric chloride activation. Polymers containing carboxyl groups can be coupled to synthetic capture probes having primary amine groups using carbodiimide coupling. Polymers carrying primary amines can be coupled to amine-containing probes with glutaraldehyde or cyanuric chloride. Many polymers can be modified with thiol-reactive groups which can be coupled to thiol-containing synthetic probes. Many other suitable methods can be found in the literature. (For review see Wong, "*Chemistry of Protein Conjugation and Cross-linking*", CRC Press, Boca Raton, FL, 1993).

A variety of capture probes can be used in the methods of the present invention. Typically, the capture probes of the present invention comprise a nucleic acid (e.g., oligonucleotide) with a nucleotide sequence substantially complementary to a nucleotide sequence region contained within the target molecule wherein the target molecule hybridizes to the capture probe. It is important to note that the capture probe is not complementary to the primer used in the primer extension sequencing reaction. The complementarity of the capture probe to the target molecule need only be sufficient so as to specifically bind the target molecule and effectuate the purification of the target molecule in the reaction mixture. Probes suitable for use in the present invention comprise RNA, DNA, nucleic acid analogs (such as PNA), modified nucleic acids and chimeric probes of a mixed class comprising a nucleic acid with another organic component, e.g., peptide nucleic acids (PNA). Capture probes can be single-stranded or double-stranded nucleic acids. Typically, the length of a capture probe will be at least 5 nucleotides in

length, more typically between 5 and 50 nucleotides, and can be as long as several thousand bases in length.

Methods for covalently attaching the capture probes described herein to polymerizable chemical groups have also been developed. When copolymerized with suitable mixtures of polymerizable monomer compounds, matrices containing high concentrations of immobilized nucleic acids can be produced. Examples of methods for covalently attaching nucleic acids to polymerizable chemical groups are found in U.S. Serial No. 08/812,105; U.S. Serial No. 08/971,845, and Rehman, F.N., *et al., Nucleic Acid Res.*, 27:649-655 (1999), the teachings of which are herein incorporated by reference in their entirety.

For some methods, it may be useful to use composite matrices containing a mixture of two or more matrix forming materials, an example is the composite acrylamide-agarose gel. These gels typically contain from 2-5% acrylamide and 0.5%-1% agarose. In these gels the acrylamide provides the chief sieving function, but without the agarose, such low concentration acrylamide gels lack mechanical strength for convenient handling. The agarose provides mechanical support without significantly altering the sieving properties of the acrylamide. In such cases, it is preferred that the nucleic acid can be attached to the component that confers the sieving function of the gel, since that component makes the most intimate contacts with the solution phase nucleic acid target.

For many applications gel-forming matrices such as agarose and cross-linked polyacrylamide will be preferred. However, for capillary electrophoresis (CE) applications it is convenient and reproducible to use soluble polymers as electrophoretic matrices. Examples of soluble polymers that have proven to be useful for CE analysis are linear polymers of polyacrylamide, poly(N,N-dimethylacrylamide), poly(hydroxyethylcellulose), poly(ethyleneoxide) and poly(vinylalcohol) as described in Quesada (*Current Opinion in Biotechnology*, 8:82-93 (1997)). These soluble matrices can also be used to practice the methods of the present invention. It is particularly convenient to use the methods found in the application U.S. Serial No. 08/812,105, entitled "Nucleic Acid-Containing Polymerizable Complex" for preparation of soluble polymer matrices containing immobilized capture probes. Another approach for attaching nucleic acid molecule

probes to preformed polyacrylamide gels found in Timofeev, et al., *Nucleic Acids Res.*, 24:3142-3148 (1996), can also be used to attach capture probes to prepolymerized soluble linear polyacrylamide.

Nucleic acids may be attached to particles which themselves can be incorporated into electrophoretic matrices. The particles can be macroscopic, microscopic, or colloidal in nature. (See Polyciences, Inc., 1995-1996 particle Catalog, Warrington, PA). Cantor, *et al.*, U.S. Patent No. 5,482,863 describes methods for casting electrophoresis gels containing suspensions or particles. The particles are linked to nucleic acids using methods similar to those described above mixed with gel forming compounds and cast as a suspension into the desired matrix form.

As defined herein, the term "nucleic acid" includes DNA (deoxyribonucleic acid) or RNA (ribonucleic acid). Nucleic acids referred to herein as "isolated" are nucleic acids separated away from the components of their source of origin (e.g., as it exists in cells, or a mixture of nucleic acids such as a library) and may have undergone further processing. Isolated nucleic acids include nucleic acids obtained by methods known to those of skill in the art. These isolated nucleic acids include substantially pure nucleic acids, nucleic acids produced by chemical synthesis, by combinations of biological and chemical methods and recombinant nucleic acids which are isolated.

"Nucleic acid analogs", as used herein, include nucleic acids containing modified sugar groups, phosphate groups or modified bases. Examples of nucleic acids having modified bases, include, for example, acetylated, carboxylated or methylated bases (e.g., 4-acetylcytidine, 5-carboxymethylaminomethyluridine, 1-methylinosine, norvaline or allo-isoleucine). Such nucleic acid analogs are known to those of skill in the art. One example of a useful nucleic acid analog is peptide nucleic acid (PNA), in which standard nucleotide bases are attached to a modified peptide backbone comprised of repeating N-(2-aminoethyl)glycine units (Nielsen *et al.*, *Science*, 254:1497-1500, (1991)). The peptide backbone is capable of holding the bases at the proper distance to base pair with standard DNA and RNA single strands. PNA-DNA hybrid duplexes are much stronger than equivalent DNA-DNA duplexes, probably due to the fact that there are no negatively charged

phosphodiester linkages in the PNA strand. In addition, because of their unusual structure PNAs are very resistant to nuclease degradation. For these reasons, PNA nucleic acid analogs are useful for immobilized probe assays. It will be apparent to those skilled in the art that similar design strategies can be used to construct other nucleic acid analogs that will have useful properties for immobilized probe assays. Probes containing modified nucleic acid molecules may also be useful. For instance, nucleic acid molecules containing deazaguanine and uracil bases can be used in place of guanine and thymine-containing nucleic acid molecules to decrease the thermal stability of hybridized probes (Wetmur, *Critical reviews in Biochemistry and Molecular Biology*, 26:227-259 (1991)). Similarly, 5-methylcytosine can be substituted for cytosine if hybrids of increased thermal stability are desired (Wetmur, *Critical reviews in Biochemistry and Molecular Biology*, 26:227-259 (1991)). Modifications to the ribose sugar group, such as the addition of 2'-O-methyl groups can reduce the nuclease susceptibility of immobilized RNA probes (Wagner, *Nature*, 372:333-335 (1994)). Modifications that remove negative charge from the phosphodiester backbone can increase the thermal stability of hybrids (Moody *et al. Nucleic Acids Res.*, 17:4769-4782 (1989); Iyer *et al. J. Biol. Chem.*, 270:14712-14717 (1995)).

As defined herein, "substantially complementary" means that the nucleic acid molecule sequence of the capture probe need not reflect the exact nucleic acid molecule sequence of the microbial target molecule, but must be sufficiently similar in identity of sequence to hybridize with the target molecule under specified conditions. For example, non-complementary bases, or additional nucleic acid molecules can be interspersed in sequences provided that the sequences have sufficient complementary bases to hybridize therewith. Generally, the degree of complementarity using short capture probes (approximately 20 nucleotides in length) is approximately greater than 95%. For longer probes significantly less complementarity is required if there are contiguous segments of from about 15 to about 20 nucleotides in length being complementary to each other.

Specified conditions of hybridization can be determined empirically by those of skill in the art. For example, conditions of stringency should be chosen that significantly decrease non-specific hybridization reactions. Stringency conditions

for nucleic acid hybridizations are explained in e.g., *Current Protocols in Molecular Biology*, Ausubel, F.M., *et al.*, eds., Vol. 1, Suppl, 26, 1991; the teachings of which are herein incorporated by reference in their entirety. Factors such as probe length, base composition, percent mismatch between the hybridizing sequences, temperature and ionic strength influence the stability of nucleic acid hybrids. Stringent conditions, e.g., moderate, or high stringency, can be determined empirically, depending on part of the characteristics of the probe and microbial target molecule.

The features and other details of the invention will now be more particularly described and pointed out in the exemplification. It will be understood that the particular embodiments of the invention are shown by way of illustration and not as limitations of the invention. The principle features of this invention can be employed in various embodiments without departing from the scope of the invention.

EXAMPLES

The following examples are not intended to limit the scope of the invention. They are provided merely to teach concrete and practical means for carrying out the invention.

EXAMPLE 1: Gel-based DNA Isolation and Elution

Sequencing reaction products were prepared using the Thermo Sequenase™ DYEnamic direct cycle sequencing kit with -21 M13 forward primer (5'-dye1-spacer-TGT*AAAACGACGGCCAGT-3' [SEQ ID No. 1]), where * indicates the position of base modification with one of four fluorescence energy transfer dyes according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). Each of four reactions was prepared by mixing 1 mL of M13mp18 single-stranded DNA (0.25 mg/mL, New England BioLabs catalog #404-C), 14 mL of distilled H_2O, and 2 mL of the manufacturer's reaction mixture. Each of the four reactions used a different dye-labeled primer, designated "dye1", "dye2", "dye3" and "dye4", and a different ddNTP nucleotide mixture. These four tubes were then placed in a thermal cycler (PTC100, MJ Research, Watertown, MA) and subjected

to 30 cycles of 95° C for 30 seconds, 45° C for 15 seconds, and 70° C for 30 seconds. The four reactions were then pooled (100 mL total volume) and 11 mL of loading buffer was added (2.5% wt/vol Xylene Cyanol, 2.5% wt/vol Bromophenol Blue, 20 mM EDTA, pH 8.0, 15% (wt/vol) Ficoll 400,000 average molecular weight in a total of 10 mL distilled H_2O).

Polyacrylamide gels for electrophoretic hybridization purification were cast in standard micropipette tips for 1-200 µL micropipettes (Fisher Brand yellow tips for Gilson P200, Fisher Scientific, Pittsburgh, PA). For the purification step, two gel tips were stacked so that the sequencing reaction could be subjected to electrophoresis through each tip sequentially in one step. (See Fig. 4A). The gel in the upper tip (10) comprised a 20 µL 5% polyacrylamide gel (29:1 monomer:bis wt/wt) cast in 1 x TBE buffer (89 mM Tris-Borate pH 8.3, 2 mM EDTA (Bio-Rad). This upper gel is designed to trap the high molecular weight M13 template DNA which has negligible electrophoretic mobility under the conditions used for capture of the extension sequencing reaction products. Removal of the high molecular weight template improves quality of sequencing results on capillary electrophoresis instruments such as the Megabase from Molecular Dynamics (Sunnyvale, CA).

The gel in the lower tip (20) is the same as that of the upper tip, except that it contains an immobilized nucleic acid molecule capture probe (5'-acrylamide-GGG ATC CTC TAG AGT CGA CCT 3' [SEQ ID NO 3]) at a concentration of 10 µM (referring to nucleic acid molecule strands). The capture probe is complementary to a sequence within the extension products that is located immediately 3' of the sequencing primer, as shown in Fig. 3.

As shown if Fig. 3, the cloned insert to be sequenced is located on the 5' side of the template region shown. Thus, as shown in the diagram, the capture probe is complementary to the extended sequencing reaction products, but not to the sequencing primer. In this way, electrophoresis of the extension sequencing reaction products through the gel of the lower tip will allow hybridization capture of the extension products without impeding electrophoresis of the excess primers through the tip.

The capture probe was modified with a 5'-acrylamide group using an acrylamide phosphoramidite (Acrydite™, Mosaic Technologies, Boston, MA). The

probe was immobilized on the gel matrix by adding it to the unpolymerized acrylamide mixture and allowing it to copolymerize directly in the gel tip.

The tips were stacked as shown in Fig. 4A, where the probe-containing tip (20) is on the bottom. The region between the two tips (30) is filled with electrophoresis buffer (1 x TBE which is 89 mM Tris-borate, pH 8.3, 2 mM EDTA), as well as the region above the upper gel tip (40). The lower tip (10) is immersed in a buffer-filled 1.5 mL microcentrifuge tube (50). Separate platinum electrodes (60) are placed in the buffer above the gel in the higher tube and in the buffer in the microcentrifuge tube (50). The upper electrode is connected to a negative lead of the power supply, while the lower electrode is attached to a positive lead.

The upper tip of the device shown in Fig. 4A was loaded with 75 μL of the pooled sequencing reaction in 15 μL aliquots every 10 minutes for one hour, while subjecting the tips to electrophoresis at an applied field of 100 V throughout the loading process. The field was applied for an additional 3 hours to ensure that all of the sequencing reaction products become trapped on the gel in the lower tip. The primers, which are not complementary to the immobilized capture nucleic acid molecule probes in the lower gel, nucleotides, and excess salts pass through the gel into the lower tube.

Following electrophoresis, the upper gel tip containing the slow-moving template was discarded and the lower gel tip (20) was then placed into a second apparatus, depicted in Fig. 4B. The lower end of the tip is placed into an electrophoresis buffer held in an ultrafiltration device (70) with a 3000 Dalton molecular weight cutoff membrane (75) on its bottom surface (Microcon 3, Amicon/Millipore, Bedford, MA). The ultrafiltration unit was partially immersed in a 1 x TBE-filled microcentrifuge tube (80) containing a positively charge platinum electrode (60). A negatively-charged electrode (60) was immersed the buffer (1 x TBE) above the gel in the tip. The ultrafiltration membrane was used to prevent the migration of the eluted sequencing reaction products onto the electrode, where they would be damaged by electrochemical reactions. To elute the sequencing reaction products, a field of 300 V was applied to the device for 3 minutes. This voltage was sufficient to elute the sequencing reaction products from the gel capture probes and cause it to collect in the ultrafiltration unit.

Fig. 5 shows the effects of varying the elution voltage. Sequencing reaction products were captured and purified by electrophoretic hybridization capture as described above. The tip was then subjected to the indicated electrophoresis conditions, and then scanned in a fluorescence imaging device (Fluoroimager 595, Molecular Dynamics, Sunnyvale, CA) to visualize the fluorescent sequencing reaction products. As seen, voltages above 250 V cause complete elution of the fluorescent sequencing reaction products.

To characterize the eluted products, samples of purified and crude sequencing reaction products were subjected to electrophoresis in a polyacrylamide gel containing a discrete layer of gel immobilized capture probe arranged as a horizontal band across the width of the gel (see "Capture layer" in Fig. 6). The gel was composed of 5% polyacrylamide (29:1 monomer:bis wt/wt), 1 x TBE. The capture layer contained the same polyacrylamide and buffer with 10 μM of the 5'-acrylamide capture probe (5'-acrylamide- GGG ATC CTC TAG AGT CGA CCT 3' [SEQ ID NO 3]). The samples were subjected to electrophoresis run at 150 Volts for 30 minutes (Fig. 6A) and 60 minutes (Fig. 6B). Lane 1 contains 15 μL of the sample that had been purified by electrophoretic capture and elution, and lane 2 contains 5 μL of the unpurified sequencing reaction product. Fig. 6A shows that the hybridization-purified product (lane 1) has been purified away from the excess primers, which are seen in the unpurified sample at the bottom of lane 2.

EXAMPLE 2: Purification of a Single DNA Product Complementary to M13mp18 Sequence

Fig. 7A provides a schematic representation of the method and device(s) described below for purifying, and optionally concentrating, products of DNA sequencing reactions. Fig. 7B provides a photograph of a capture probe-gel-tip after electrophoresis and prior to elution of the captured nucleotide sequence.

Fig. 8 provides a photograph of a gel showing the results of purification of a desired oligonucleotide sequence from a DNA sequencing reaction that included primers, salts, DNA template, unincorporated nucleotides, and dye terminators. First the DNA sequencing reaction was purified by gel-loading tip to provide a crude sample, then the crude sample was purified by capture probe-gel-tip. Lane 1 shows

the pattern provided prior to purification. Lane 2 provides the pattern seen after purification using a gel-loading tip. Removal of DNA template is demonstrated. Lane 3 shows the pattern seen after purification using a capture probe-gel-tip. Localization of the desired sequence in the absence of DNA template is demonstrated.

A. Preparation of a Separation Device With and Without an Immobilized Capture Probe

Preparation of the separation device containing Acrydite™ oligonucleotide gel was performed as follows. A Hybrigel solution was prepared from stock solutions of 40% acrylamide (29:1 acrylamide monomer:bis-acrylamide) and 10x TBE (90mM Tris-Borate-EDTA buffer pH 8.3; reagents were purchased from Biorad Laboratories, Inc.; Hercules, CA). The Acrydite™ oligonucleotide for the capture probe was synthesized using oligonucleotides (obtained from Operon Technologies, Inc.; Alameda, CA) and acrylamide phosphoramidte (Acrydite polymer available from Mosaic Technologies, Inc.; Boston, MA) according the method disclosed in U.S. Patent No. 5,641,658 and Kenney, Ray, and Boles, BioTechniques 25, 516-521 (1998), the disclosure of each of which is incorporated herein by reference in its entirety). The Hybrigel contained 5% acrylamide (29:1), 1 x TBE and 10 μM AcryditeTM oligonucleotide capture probe (SEQ ID NO 4) having the following sequence:

5'-acrylamide GCT GAG ATC TCC TAG GG 3' (SEQ ID NO 4)

The selected capture probe for this example has a sequence that is complementary to a portion of the polylinker of vector M13mp18 which provides the target molecule, a product of a DNA sequencing reaction, but the capture probe does not include any of the primer sequence. When 10% ammonium persulfate and N,N,N',N'-tetramethyl-ethylenediamine (TEMED; BioRad, Hercules, CA) were added to the Hybrigel solution, polymerization was rapid (within 2 minutes).

To prepare the probe-gel-tip, 1μl of 10% ammonium persulfate and 0.5μl of TEMED were added to 200μl of Hybrigel solution. 10μl of the polymerizing

Hybrigel oligonucleotide containing solution were quickly pipetted into a 200µl tip and allowed to polymerize. The probe-gel-tips were made 8 or 12 at a time using a multipipeting device. For storage, probe-gel-tips were ejected into eppendorf tubes containing approximately 0.3 ml of 1x TBE. Care must be taken not to dislodge the gel from the tip. Then the probe-gel-tips were overlaid with 150µl of 1x TBE.

Gel-loading tips were used to remove template DNA. Gel-loading tips were prepared containing acrylamide (29:1) only (i.e. no Acrydite capture probe). The gel-loading-tip was prepared as described above using a solution containing 5% acrylamide (29:1), 1 x TBE, made from stock solutions of 40% acrylamide (29:1 monomer:bis) and 10x TBE. 10% ammonium persulfate and TEMED were added to the solution and 200 µl of the final solution was pipetted into each tip. Gel-loading-tips may also be stored as described above.

B. Preparation of a DNA Sequencing Reaction Product and Capture Probes

PE-Applied Biosystems sequencing reaction products were prepared following the protocol of PE Applied Biosystems BigDye Primer Cycle Sequencing Kit (available from PE-Applied BioSystems) with the -21 M13 forward primer in a GeneAmp 2400 using the cycling conditions recommended by PE Applied Biosystems. Vector M13mp18 was used. A DNA segment having a known sequence was inserted after the primer site. Extension products were prepared. Capture probes were made to the region between the primer and the inserted DNA. The capture probes capable of hybridizing to extension products of the forward primer were selected. The capture probes comprise an oligonucleotide synthesized with Acrydite TM at the 5' end.

Alternatively, the DYEnamic ET Terminator Cycle Sequencing Kit from Amersham-Pharmacia may be used.

C. Capture of DNA Sequencing Extension Product

Electrophoretic capture and separation of a chosen extension product (target in test sample) were performed as follows: 10µl of sequencing reaction solution (i.e. 1/2 of one reaction) that contained primers, salt, unincorporated

nucleotides, dye terminators, template DNA, and the target DNA extension products synthesized from the template DNA were added to 1μl of 10x ficoll loading buffer (35% ficoll 400, 0.1% bromophenol blue, 0.1% xylene cyanol, 100mM EDTA) to provide a sample solution. The sample solution was layered onto the surface of the gel in the gel-loading-tip, (i.e., no Acrydite capture probe), to remove template DNA. This tip was stacked above a probe-gel-tip, a Hybrigel containing tip, as shown in Fig. 7A. A small volume of electrophoresis buffer was layered onto the surface of the gel in the probe-gel-tip and the gel-loading-tip was placed in contact with the electrophoresis buffer. Thus, a liquid connection was formed between the two gels allowing for an electrical connection when the electrodes and power source were in place.

After the sample solution was loaded onto the surface of the gel of the gel-loading-tip, both tips were placed into an ependorf tube containing 1x TBE, electrophoresis buffer. Platinum electrodes were placed in the electrophoresis buffer above and below the stacked gel tips and a voltage of 100V was applied for 10 minutes. The upper electrode was connected to the negative lead of the power supply, while the lower electrode was attached to the power supply's positive electrode. The gel-loading tip thus provided a partially purified sample solution for introduction into the probe-gel-tip. The smaller DNA fragments pass into the buffer and then into the probe-gel-tip more rapidly than the DNA template and dye which are retained in the gel-loading-tip

The electrophoresis buffer remaining above the probe-gel-tip gel surface was removed. The electrophoresis buffer was replaced with 4μl of formamide loading dye (5:1 deionized formamide, 25mg/ml blue dextran, 25mM EDTA). The temperature in the gel in the probe-gel-tip was raised to 55 ^{0}C to facilitate detachment of the target oligonucleotide sequence from the capture probe by placing the eppendorf tube containing the lower buffer reservoir into a drybath. (VWR). A clean second gel-loading tip with a 30% acrylamide gel containing 5 % acrylic acid was lowered into the formamide loading dye. A platinum electrode was placed in the top gel-loading tip. The direction of the current was reversed to drive the oligonucleotide released from the hybridization complex upwards. One minute at 40V was sufficient to drive the oligonucleotide out of the probe-gel-tip and into the

formamide loading dye. A 4 µl sample was removed by pipet and retained for sequence analysis. After electrophoresis, the gel in the probe-gel-tip was visualized using a Molecular Dynamics Fluorimager 595. The results shown in Fig. 7B demonstrate that capture occurs at the upper surface of the gel in the probe-gel-tip.

D. Analysis of Sequencing Purification by Hybrigel Assay

Glass plates for a vertical polyacrylamide minigel (10 x 10 cm, 0.75 mm spacers) were assembled and the sandwich was filled approximately half way with 20% acrylamide (29:1; Bio-Rad), 1x TBE (90 mM Tris-borate buffer, pH 8.3, 2 mM EDTA). Polymerization was initiated by inclusion of 10% aqueous ammonium persulfate (APS) and TEMED at 1/100th and 1/1000th gel volume, respectively. For gels containing one capture layer, 600 µl of gel solution (20% polyacrylamide, 1x TBE, 4 µl 10% APS and 4 µl 10% TEMED) containing Acrydite-labeled oligonucleotide at a final concentration of 10 µM were polymerized. After polymerization of the capture layer, the remaining space in the plate sandwich was filled with a 5% gel. This composite gel was then assembled in a minigel apparatus containing 1x TBE and subjected to electrophoresis at 100-150 V for ~45 min. After electrophoresis, the gel was visualized using a Molecular Dynamics Fluorimager 595. The results confirm that the sequence captured by the Hybrigel probe is the complement of the template DNA.

E. Automated Sequencing of the Captured Oligonucleotide

Following the procedures described above and analyzing the oligonucleotide sequence purified by the inventive device with an automated sequencer, repeated experiments with standard vectors have demonstrated that the accuracy of sequencing of the first 500 nucleotides is always >99%. The readable sequence extends to at least 750 nucleotides.

EXAMPLE 3: Simultaneous Separation of Multiple DNA Sequencing reaction products

This example demonstrates that the method of the invention is useful for sequencing an oligonucleotide insert replicated in a plasmid. Both forward and

reverse primers are used as illustrated in Fig. 9A. A plasmid with a large insert was sequenced simultaneously using two primers in one reaction vessel. Two probe-gel-tips each containing a different capture probe were arranged in tandem. The probe sequences were designed based upon the forward and reverse primers used. From the slab gel illustrated in Fig. 9C, purification of the two oligonucleotide targets in the test sample as compared to the crude product is demonstrated. Note that the sequence of the reverse product (right) shows no contamination with the forward sequence. Note also that DNA template contamination is removed.

A. Preparation of Separation Device With and Without Immobilized Capture Probe

Gel-loading tips were prepared as described in Example 2. Probe-gel-tips were prepared as described in Example 2 except that one was provided with a forward primer probe and the other was provided with a reverse primer probe as shown in Fig. 9A. Thus, two separate Hybrigel probe-gel-tips were made, one with acrydite oligonucleotide (SEQ ID NO 5) and the other with acrydite olgonucleotide (SEQ ID NO 6). Plasmid p698 which is plasmid vector pGEM3Zf(-) (Promega Biotech, WI) with a 3.8kb insert in the Xba I site of the polylinker was used. The two probes derive from sequence on either side of the Xba I site within the polylinker. SEQ ID NO 5 is complementary to, and therefore capable of, capturing sequencing reaction products made using the reverse primer. Similarly, SEQ ID NO 6 can capture sequence from the forward primer.

SEQ ID NO 5 5'acrylamide –TGCAGGCATGCAAGCTT

SEQ ID NO 6 5'acrylamide –GGGTACCGAGCTCGAATTC

Thus, each oligonucleotide primer probe is synthesized with Acrydite at the 5' end and is capable of hybridizing to an extension product. Each capture probe sequence is both complementary to an extension product sequence and to the primer. Each capture probe sequence is specific for a particular vector derived from the region between the primer site and the insert DNA.

B. Purification of Products of Multiple Reactions

Probe2-gel-tip, containing SEQ ID NO 6 was placed in tandem (stacked) with a probe3-gel-tip, containing SEQ ID NO 5 as is illustrated in Fig. 9A. Running electrophoresis buffer was placed on the upper surface of probe3-gel-tip to provide electrical contact and to prevent drying. The test sample from a multiplex sequencing reaction was electrophoresed through both probe2-gel-tip and probe3-gel-tip. Fig. 9B illustrates capture in the two separate tips by the two distinct probes.

EXAMPLE 4: Elution of Target from Capture Probe using a Temperature Gradient

The stability of the hybridization complex is dependent on temperature. A vertical slab gel containing a layer of Acrydite™ capture probe sandwiched between layers of gel without capture probe was made. The gel for the upper and lower layers was that used for the gel-loading-tip. The Acrydite probe layer was made as described in Example 2 for the probe-gel-tip using the capture probe sequence SEQ ID NO 5. The sample in this case was a fluorescent oligonucleotide with a complementary sequence to SEQ ID NO 5. The same sample was loaded in each well. The whole gel was subjected to a temperature gradient using an aluminum backplate and two water baths. The gel temperature on the left is 23 ^{0}C. The temperature increased across the gel up to 53 ^{0}C on the right. At low temperature the target was efficiently captured at the top of the capture layer. As the temperature was increased, target capture was inhibited until the sample runs right through the layer (see gel image in Fig. 10). The transition temperature, i.e. the temperature at which the target stops ceases to be captured is related to the Tm but that was not the only factor found to be involved.

EXAMPLE 5: Temperature and Capture Probe Size Dependence of Sequence Elution

To define temperature conditions for capture and elution of sequencing reaction products the experiment shown in Fig. 11 was performed. This experiment demonstrates that the temperature of elution is affected by the size of the capture

probe. The temperature of elution was shown to be affected by the size of the capture probe. The five panels shown in Fig. 11 show the same vertical slab gel run at five different temperatures starting with 23 ^{0}C wherein five different capture probes were used. The number of bases in each probe sequence was 13, 15, 17, 19, and 21 nucleotides as indicated in Fig. 11 (from left to right). An M13 sequencing reaction (Dynamic™ ET) was loaded in each lane. The 13mer capture probe did not effectively captured the sequencing reaction products at 23 ^{0}C. All the others capture probe (i.e., having lengths 15, 17, 17, and 21) captured the sequencing reaction products at 23 °C. At 30 ^{0}C, the 15mer released the sequence while the 17mer started to release at 35 ^{0}C and so on until at 50 ^{0}C all target sequences came off. These results illustrate temperature conditions for capture and elution of sequencing reaction products.

The 17mer Acrydite probe was chosen as the capture probe in the standard procedure with elution occurring at 45^{0}C.

EQUIVALENTS

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A method for purifying target molecules from a primer extension sequencing reaction using a purification device comprising the following steps:

(a) introducing the primer extension sequencing reaction mixture into a purification device comprising an electrophoretic medium, wherein the electrophoretic medium contains immobilized capture probes;

(b) subjecting the electrophoretic medium of step (a) to an electric field resulting in the electrophoretic migration of one, or more, target molecules into at least one region of the electrophoretic medium containing immobilized capture probes, wherein the target molecules bind to the immobilized capture probes, and

(c) collecting the target molecules of step (b).

2. The method of Claim 1, wherein the purification device is a microtiter plate.

3. The method of Claim 2, wherein the microtiter plate comprises multiple wells.

4. The method of Claim 3, wherein the number wells contained within the microtiter plate is selected from the group consisting of: 6, 12, 48, 96 and 384.

5. The method of Claim 1 wherein in step (c), a sufficient voltage is applied to release the target molecule from its complementary capture probe, and wherein the target molecule continues electrophoretic migration under the

influence of an electric field and exits the electrophoretic medium, and wherein it collects in a collecting chamber.

6. The method of Claim 5, wherein the polarity of the electric field is reversed, wherein the released target molecule will migrate back toward the test sample receptacle and wherein it is subject to collection.

7. The method of Claim 1, wherein the capture probe is a nucleic acid molecule.

8. The method of Claim 7, wherein the capture probe is complementary to the primer extension sequencing reaction product.

9. The method of Claim 8, wherein the capture probe is from about 20 to about 2000 nucleotides in length.

10. A method for purifying multiple primer extension sequencing reaction products which are formed by synthesizing primer extension sequencing reaction products using both a first-end and a second-end of a DNA template comprising the following steps:

(a) introducing the primer extension sequencing reaction mixture into a purification device comprising at least two cartridges, wherein each cartridge comprises an electrophoretic medium containing a unique set of immobilized capture probes;

(b) subjecting the electrophoretic media of step (a) to an electric field resulting in the electrophoretic migration of one, or more, primer extension sequencing reaction products into at least two cartridges each containing an electrophoretic medium, wherein each medium contains a unique set of immobilized capture probes, and wherein the primer extension sequencing reaction products bind to the appropriate immobilized capture probe, and

(c) collecting the target molecules of step (b).

11. The method of Claim 10, wherein the purification device is a microtiter plate.

12. The method of Claim11, wherein the microtiter plate comprises multiple wells.

13. The method of Claim 12, wherein the number wells contained within the microtiter plate is selected from the group consisting of: 6, 12, 48, 96 and 384.

14. The method of Claim 10 wherein in step (c), a sufficient voltage is applied to release the target molecule from its complementary capture probe, and wherein the target molecule continues electrophoretic migration under the influence of an electric field and exits the electrophoretic medium, and wherein it collects in a collecting chamber.

15. The method of Claim 14, wherein the polarity of the electric field is reversed, wherein the released target molecule will migrate back toward the test sample receptacle and wherein it is subject to collection.

16. The method of Claim 10, wherein the capture probe is a nucleic acid molecule.

17. The method of Claim 16, wherein the capture probe is complementary to the primer extension sequencing reaction product.

18. The method of Claim 17, wherein the capture probe is from about 20 to about 2000 nucleotides in length.

19. A kit for purifying a primer extension sequencing reaction, comprising a electrophoretic medium, wherein the electrophoretic medium contains a capture probe, or a set of capture probes, having a substantially

complementary sequence of at least 5 nucleotide bases in length to a portion of at least one primer extension sequencing reaction product.

20. The kit of Claim 19, further comprising a test sample receptacle and a collecting chamber at opposite ends of the electrophoretic medium.

21. The kit of Claim 20, wherein the capture probe is from about 20 to about 2000 nucleotide bases in length.

22. A kit for purifying multiple primer extension sequencing reaction, comprising multiple electrophoretic media, wherein each electrophoretic medium contains a capture probe, or set of capture probes, having a substantially complementary sequence of at least 5 nucleotide bases in length to a portion of at least one primer extension sequencing reaction product.

23. The kit of Claim 22, wherein the electrophoretic media are segregated in wells of a microtiter plate.

24. The kit of Claim 23, further comprising multiple test sample receptacles and multiple collecting chambers, wherein a sample loading receptacle and a sample collecting chamber is located at opposite ends of each electrophoretic medium.

25. The kit of Claim 24, wherein the capture probe is from about 20 to about 2000 nucleotide bases in length.

26. The kit of Claim 23, wherein each electrophoretic medium contains the same capture probe or set of capture probes.

27. The kit of Claim 23, wherein each electrophoretic medium contains a different capture probe, or set of capture probes.

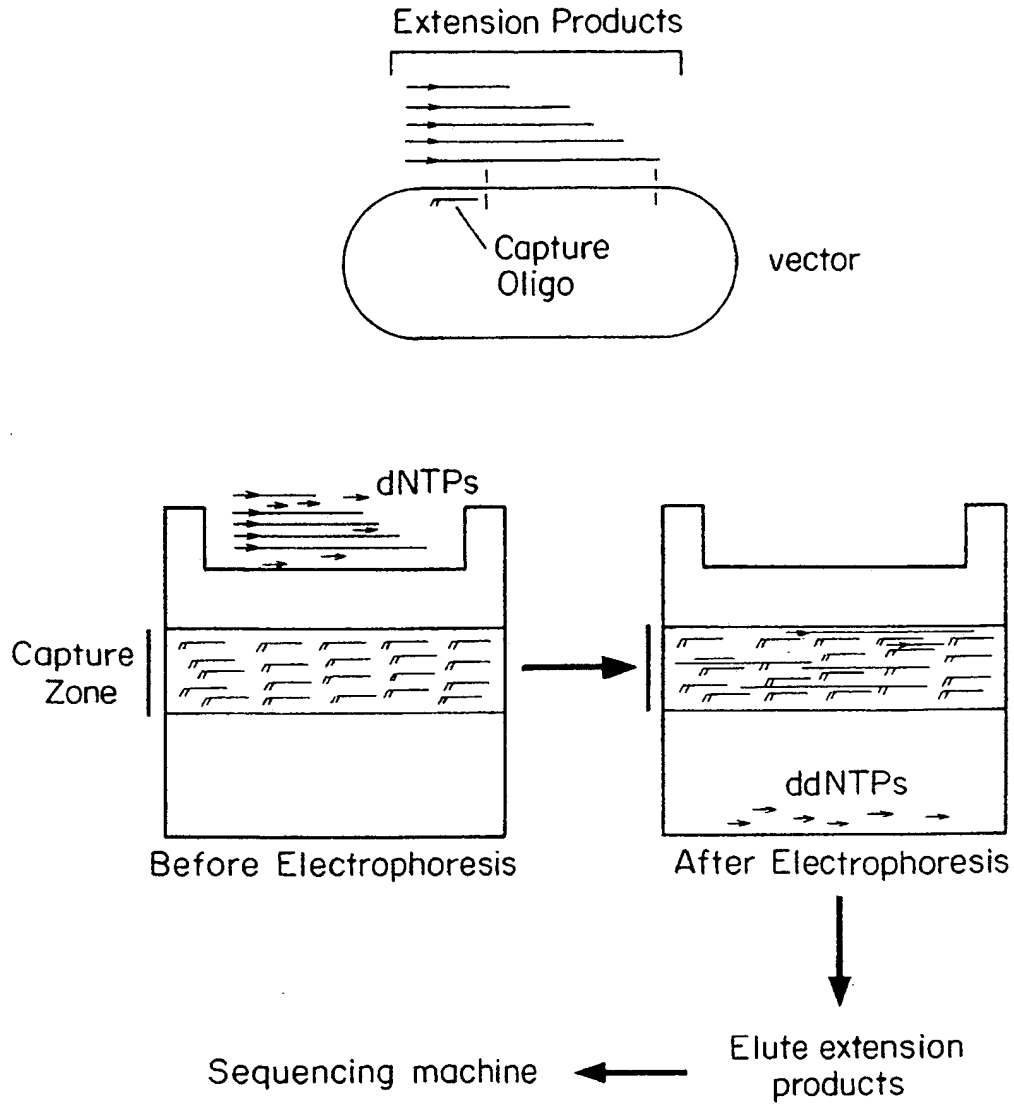

Extension Products
Capture Oligo
vector
dNTPs
Capture Zone
Before Electrophoresis
ddNTPs
After Electrophoresis
Elute extension products
Sequencing machine

FIG. 1

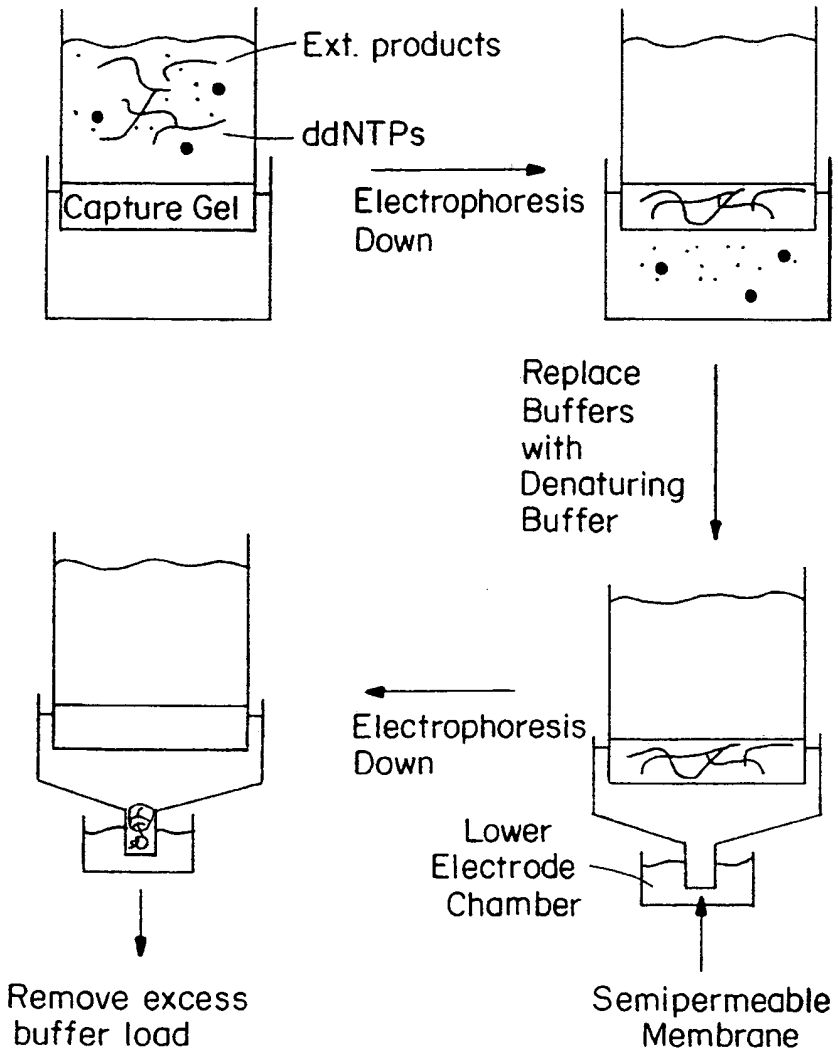

Ext. products
ddNTPs
Capture Gel
Electrophoresis Down
Replace Buffers with Denaturing Buffer
Electrophoresis Down
Lower Electrode Chamber
Remove excess buffer load
Semipermeable Membrane

FIG. 2

5' -TGTAAAACGACGGCCAGT-3' (M13 -21 forward primer)
3' -AACATTTGCTGCCGGTCACGGTTCGAACGGTACGGACGTCCAGCTGAGATCTCCTAGGG- 5' (template)
Capture probe 3' -TCCAGCTGAGATCTCCTAGGG-ac-5'

FIG. 3

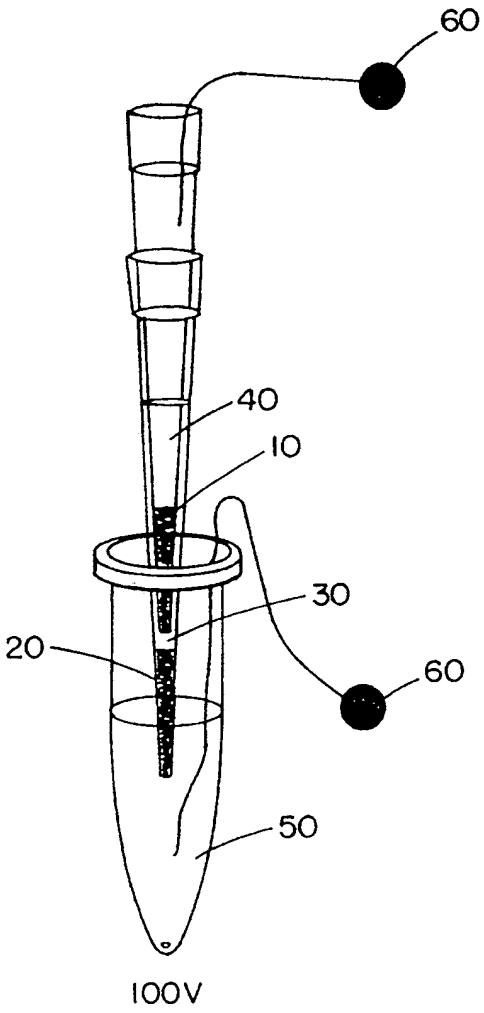


FIG. 4A

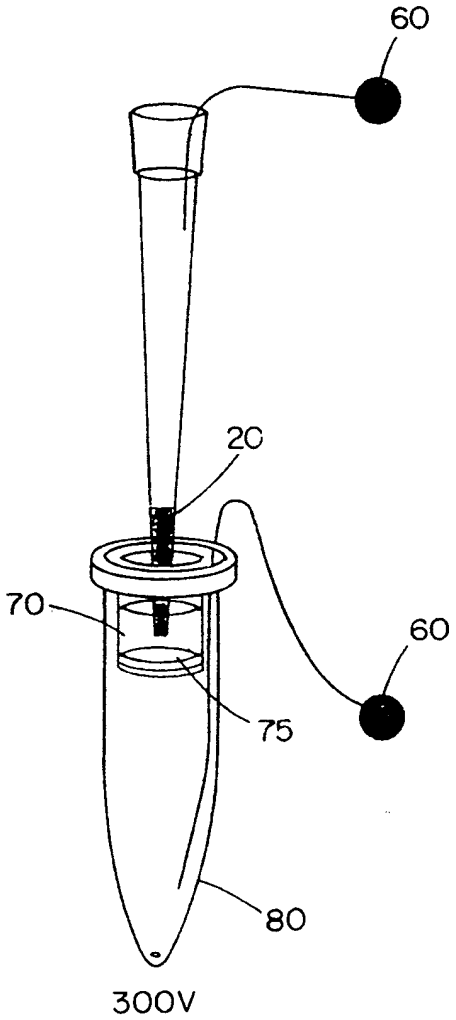


FIG. 4B

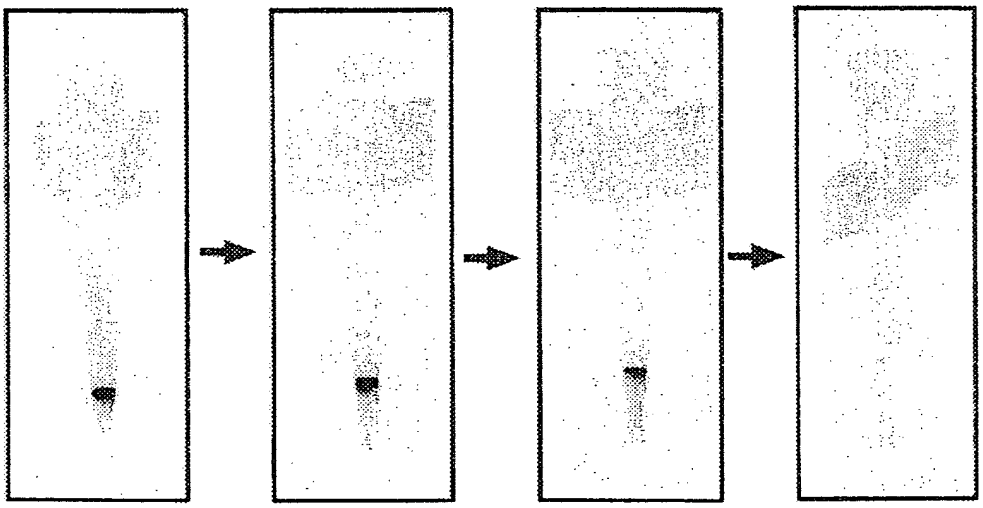


FIG. 5

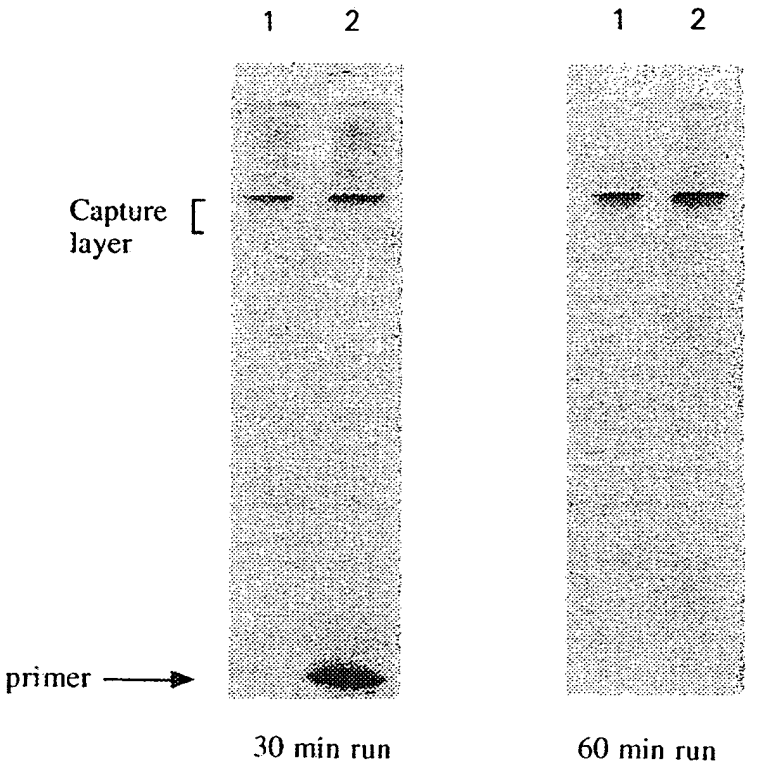


FIG. 6A FIG. 6B

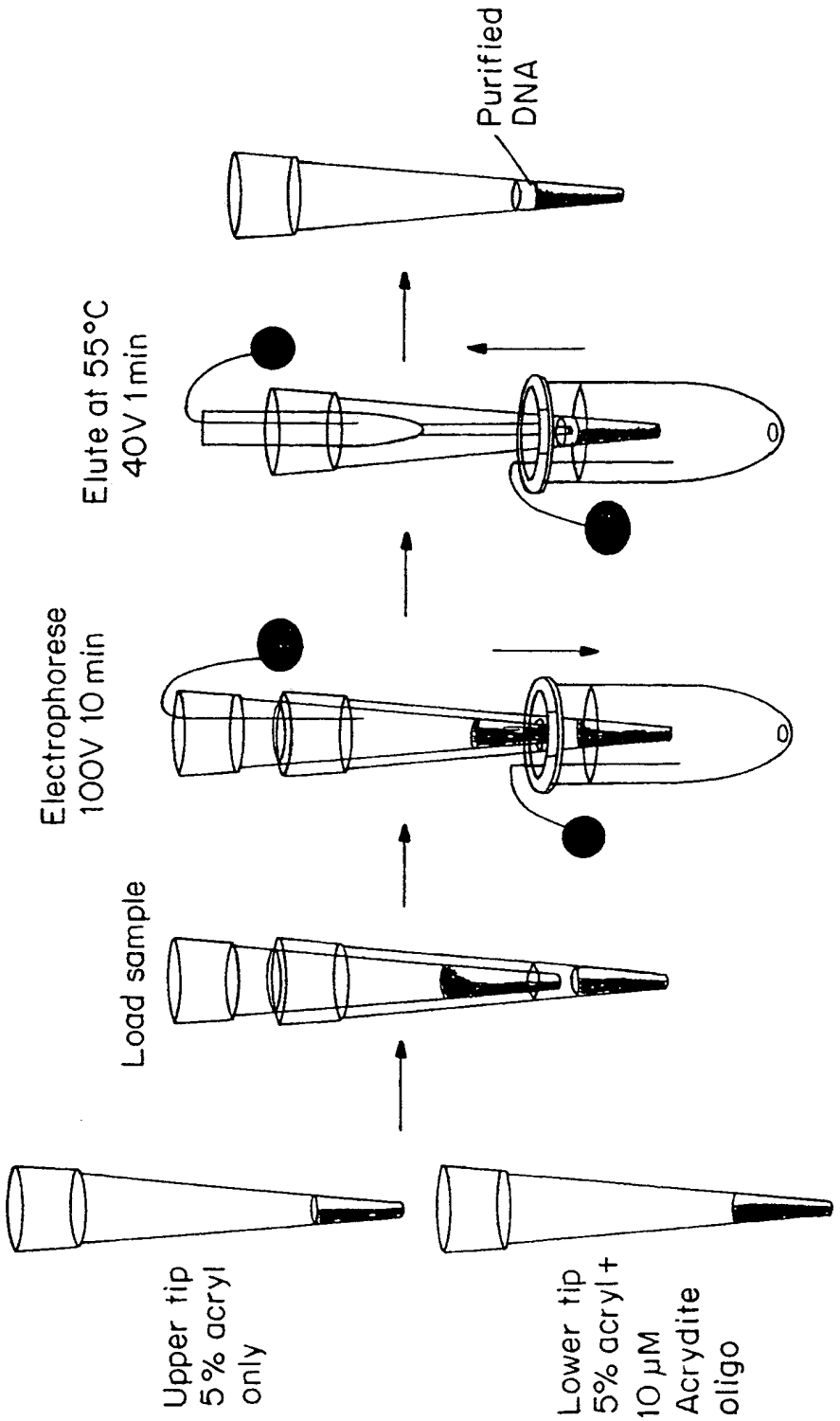
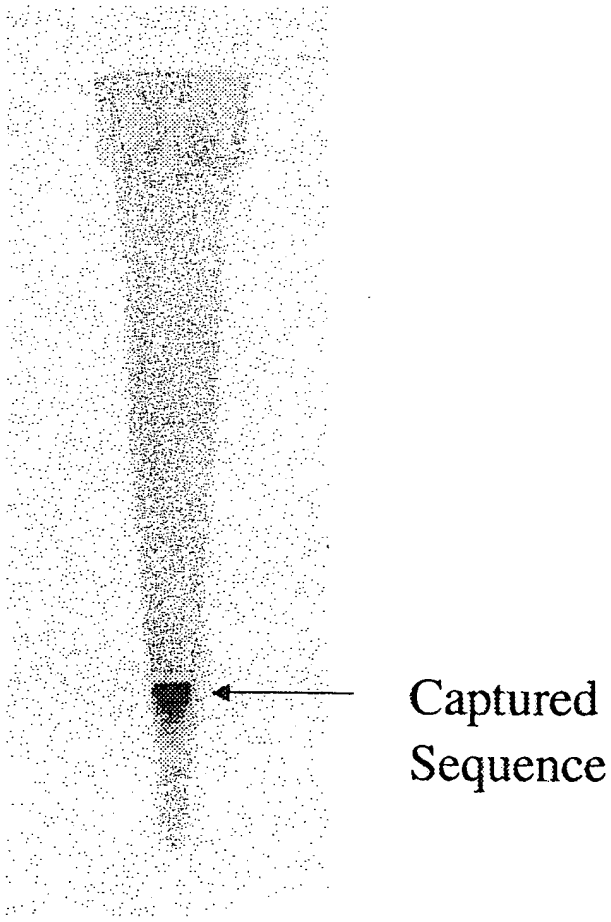

Purified DNA
Elute at 55°C 40V 1 min
Electrophorese 100V 10 min
Load sample
Upper tip 5% acryl only
Lower tip 5% acryl + 10 µM Acrydite oligo

FIG. 7A

FIG. 7B



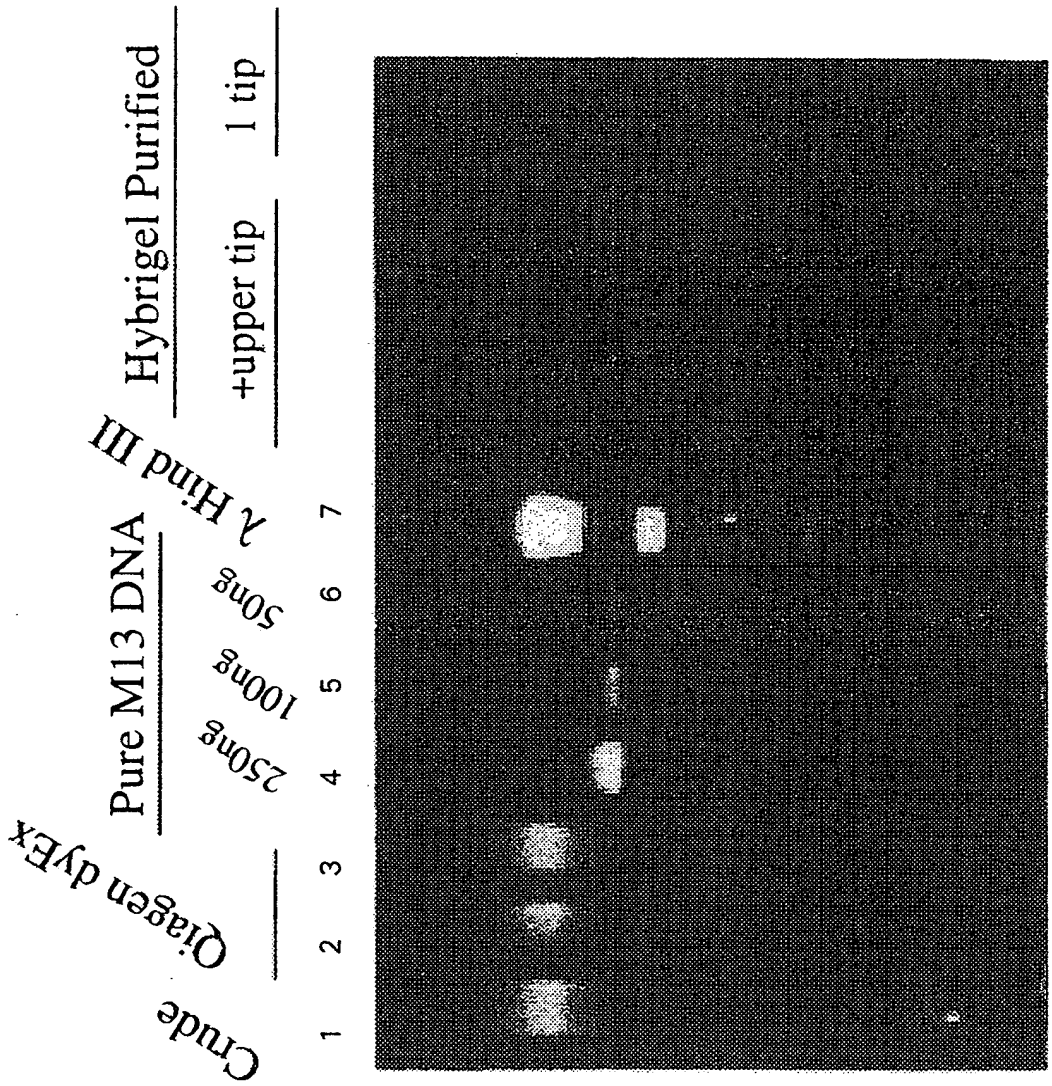

Crude
Qiagen dyEx
Pure M13 DNA
250ng
100ng
50ng
λ Hind III
Hybrigel Purified
+upper tip
1 tip
1
2
3
4
5
6
7

FIG. 8

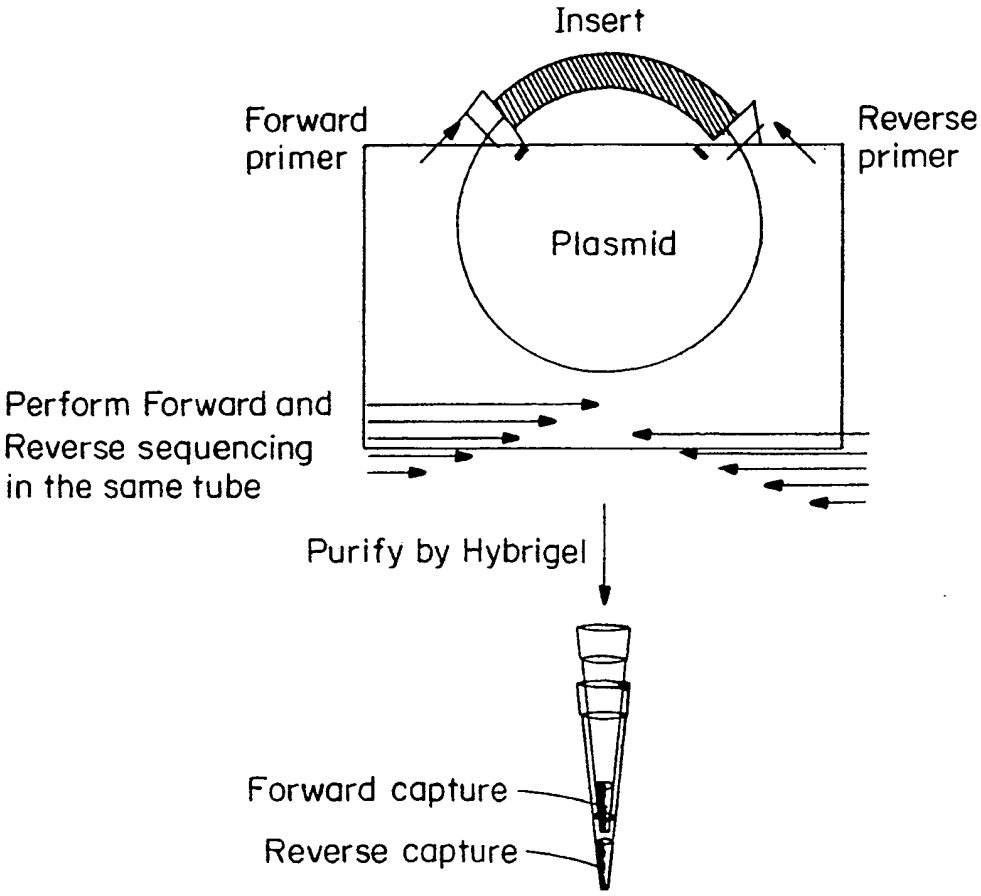

Insert
Forward primer
Reverse primer
Plasmid
Perform Forward and Reverse sequencing in the same tube
Purify by Hybrigel
Forward capture
Reverse capture

FIG. 9A

FIG. 9B

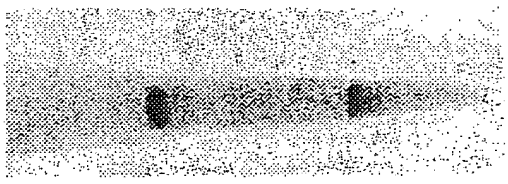


FIG. 9C

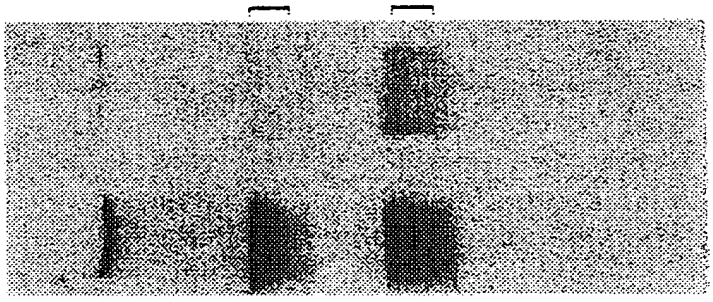


FIG. 9D

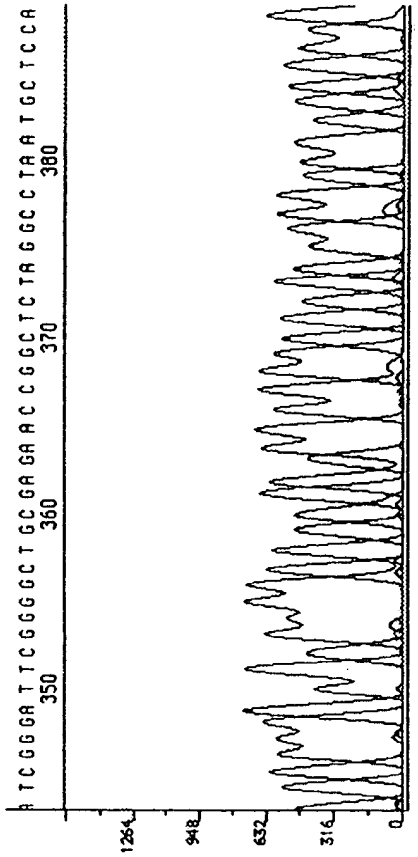


FIG. 10

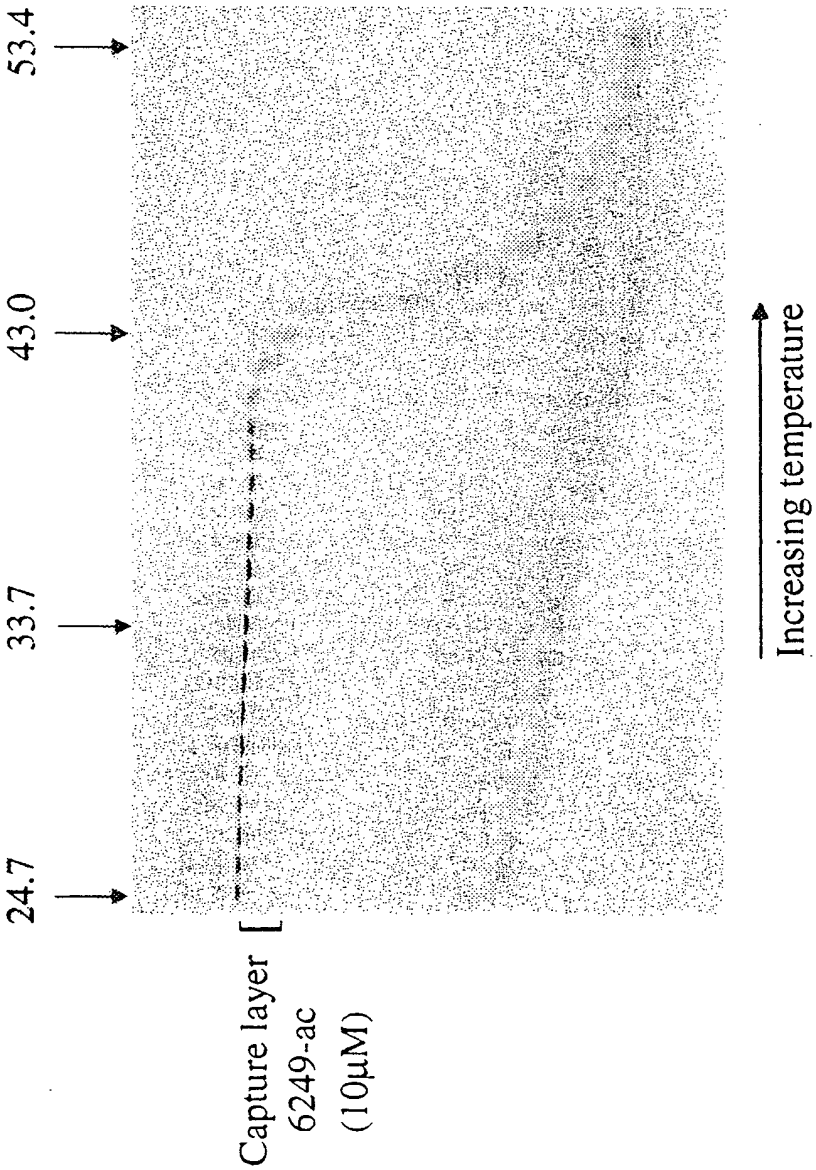
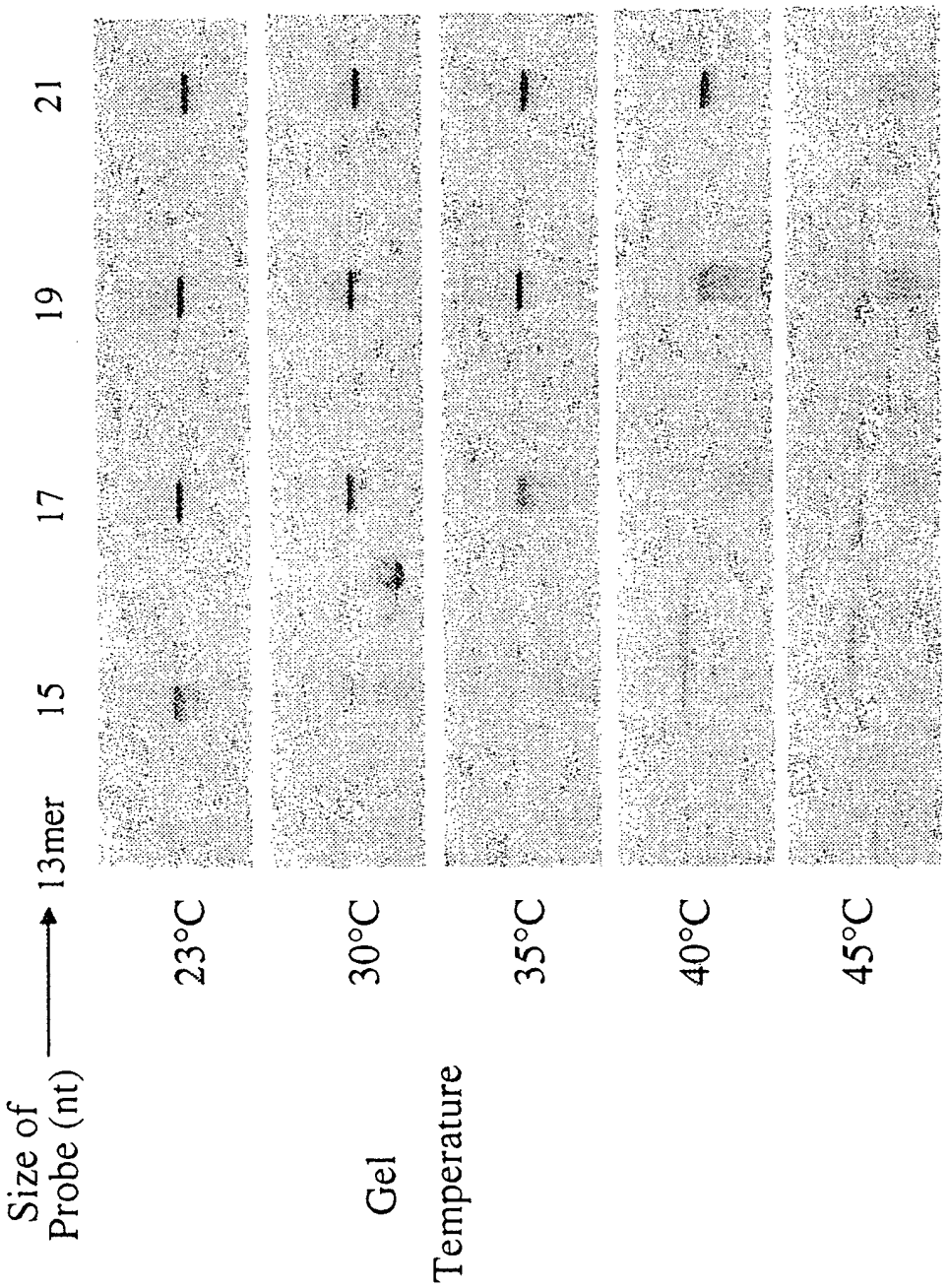

53.4
43.0
33.7
24.7
Capture layer
6249-ac
(10μM)
Increasing temperature

FIG. 11


Size of Probe (nt)
13mer
15
17
19
21
Gel Temperature
23°C
30°C
35°C
40°C
45°C